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L2	2709020	S	CANCER OR TUMOR OR TUMOUR
L3	897540	S	CARCINOMA
L4	279	S	L1 AND (L2 OR L3)
L5	161	DUP REM	L4 (118 DUPLICATES REMOVED)
L6	416	S	((SHAH G.V.) OR (SHAH G V) OR (SHAH, GIRISH V.) OR (SHAH, G.)
L7	0	S	L6 AND L1
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L9	25	DUP REM	L8 (20 DUPLICATES REMOVED)
L10	69446	S	CALCITONIN
L11	19	S	L10 AND L1 AND (L2 OR L3)
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3. Chien, J. et al. Oncogene (1999) 18(22): 3376-3382
4. Wong, E.C.C. et al. Proc. Amer. Assoc. for Cancer Res. (1997) 38: 288
5. Rayford, W. et al. Prostate (1997) 30(3): 160-166
6. Xue-Zhang, Q. et al. Endocrine (1995) 3(6): 445-451
7. Shah, G.V. et al. Endocrinology (1994) 134(2): 596-602
8. Rayford, W. et al. J. of Urology (1994) 151(5 suppl): 490A
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10. Shah, G.V. et al. Prostate (N.Y.) (1992) 21(2): 87-97
11. Sagol, O. et al. Annals of Medical Sciences (1999) 8(1): 14-21
12. Sussenot, O. et al. Prostate (1998) 36(suppl. 8): 43-51
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16. Esik, O. et al. European J. Gynaecological Oncology (1994) 15(3): 211-216

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ROLE OF STIMULATORY GUANINE NUCLEOTIDE BINDING PROTEIN ($G_{S\alpha}$) IN PROLIFERATION OF PC-3M PROSTATE CANCER CELLS

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Previous studies have shown that calcitonin-like immunoreactive substances are secreted by primary prostate cells. Furthermore, exogenously added calcitonin stimulates proliferation of androgen-responsive LnCaP cells. To examine the possible effect of calcitonin on growth of invasive prostate cancer cells, we tested its effects on proliferation of PC-3M cells. Calcitonin stimulated DNA synthesis of PC-3M cells in a dose-dependent fashion, and also stimulated adenylyl cyclase and protein kinase C activities. To further delineate the role of these signaling cascades in proliferation of PC-3M prostate cancer cells, we selectively activated these pathways by transfecting cDNAs expressing constitutively active forms of either $G_s\alpha$ ($G_s\alpha$-QL) or $G_q\alpha$ ($G_q\alpha$-QL). cDNAs expressing wild-type forms of G-proteins ($G_s\alpha$-WT and $G_q\alpha$-WT) were used as vehicle controls. $G_q\alpha$-QL transfectants exhibited growth inhibition and terminal differentiation. Those expressing $G_s\alpha$-QL exhibited a dramatic increase in growth rate. $G_s\alpha$-QL transfectants displayed an almost 3-fold increase in [^{3}H]-thymidine incorporation and over a 4-fold increase in growth rate when compared with parental PC-3M cells or those expressing wild-type $G_s\alpha$ ($G_s\alpha$-WT). The growth-promoting action of $G_s\alpha$-QL could not be mimicked by either 8-bromo cAMP or forskolin. However, nifedipine, a calcium channel antagonist, potently and selectively inhibited DNA synthesis in $G_s\alpha$-QL transfectants. These results suggest that the growth-promoting actions of $G_s\alpha$ on PC-3M cells may be mediated by nifedipine-sensitive proliferative events.

Key words: *G-protein-cell proliferation; prostate cancer*

The growth of malignant prostate is usually androgen dependent in its early stages.[1,2] However, the tumor progresses to an androgen-independent form where proliferation of tumor cells is predominantly supported by autocrine/paracrine factors.[1,3,4] The presence of several mitogenic substances such as vasoactive intestinal peptide (VIP)-like, thyroid-stimulating hormone (TSH)-like, calcitonin-like, bombesin-like peptides, serotonin, dopamine, adrenergics and muscarinic cholinergics has been reported in prostate epithelium.[5–7] Previous findings from this laboratory have shown that primary prostate cells secrete calcitonin-like immunoreactive substance(s), and that epithelial cells derived from prostate carcinoma (PC) secrete several-fold greater amounts of this substance(s) than those derived from benign prostatic hyperplasia (BPH).[8] Exogenously added calcitonin stimulates cyclic, 3′-5,adenosine monophosphate (cAMP) accumulation, increases cytoplasmic Ca^{2+} transients and causes a significant increase in DNA synthesis and invasiveness of LnCaP human prostate cancer cells.[9,10] Similar actions of other neuroendocrine factors such as VIP, bombesin, muscarinic cholinergics and serotonin on PC cells have been demonstrated.[11–13]

Among the established human prostate cancer cell lines, PC-3M, a metastatic variant of PC-3 cells, and DU-145 cell lines are poorly differentiated and grow aggressively in an androgen-independent fashion.[14,15] In contrast, LnCaP prostate cancer cells are indolent, androgen responsive and well differentiated.[16] Our previous studies demonstrated mitogenic actions of calcitonin on indolent LnCaP cells. Our present studies tested the hypothesis that calcitonin also plays an important role in androgen-independent growth of aggressive PC-3M cells, and the signaling pathways activated by calcitonin such as $G_s\alpha$- and $G_q\alpha$-mediated mechanisms are crucial for growth of aggressive prostate cancer cells.

MATERIAL AND METHODS

Cell culture

Dr. I. Fidler (Anderson Cancer Center, Houston, TX) kindly provided the PC-3M cell line. The cells were maintained in a complete medium (RPMI 1640 supplemented with L-glutamine, 5% FCS, 12% horse serum, 50 U/ml penicillin, 50 μg/ml streptomycin).

^{3}H-thymidine incorporation

PC-3M cells were seeded at a density of 10,000 cells per well and incubated overnight in the complete medium. Next day, the medium was replaced with the serum-free incubation medium (RPMI 1640 supplemented with L-glutamine, 0.3% BSA, 20 mM HEPES, 50 U/ml penicillin, 50 μg/ml streptomycin). The incubation was continued for 24 hr in some experiments as described in the Results section. On the following day, the cells were rinsed and incubated in serum-free medium containing the agents (various concentrations of calcitonin or other agents) and 0.5 μCi of ^{3}H-thymidine for 24 hr. At the end of the incubation, the cells were washed 3 times with PBS-1 μM thymidine and incorporated radioactivity was determined in TCA-precipitable fraction.[9]

Stock calcitonin solution (100 μM) was made by dissolving the peptide in distilled water and the working dilutions were made in the serum-free incubation medium. Vehicle controls received an equivalent volume of incubation medium. Similarly, Rp.cAMP was dissolved in the incubation medium. Stock solutions (1 mM) of nifedipine and verapamil were made in DMSO and were subsequently diluted in the incubation medium.

Adenylyl cyclase assay

Membrane fraction of PC-3M cells was obtained and adenylyl cyclase activity was determined as previously described.[17] In brief, PC-3M cell membranes were resuspended in a dilution buffer (25 mM HEPES, 250 mM sucrose, pH 7.4, to a final concentration of 200 μg/ml) and adenylyl cyclase activity was determined indirectly by measuring the conversion of cAMP from [α^{32}]-ATP.[17] Each assay tube contained 4 μg of membrane protein in an assay buffer containing 0.2 μCi [α^{32}P]-ATP. The basal control group contained vehicle alone, whereas the experimental group received various concentrations of calcitonin (1, 10 and 100 nM). In a positive control group, 25 μM forskolin was added to the protein-ATP mix. The heat-inactivated enzyme (PC-3M membranes)

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served as a blank control group. Each group, in triplicate, was incubated in a 37°C water bath for 15 min. The reaction was terminated by the addition of a carrier buffer (5 mg/ml cAMP, 0.5 N HCl). The reaction mixtures were transferred individually to a SpinZyme acidic alumina device (Pierce Laboratories, Rockford, IL), which binds to cyclic nucleotides under acidic conditions. The non-cyclic form of nucleotides is removed by washing with 3 × 200 μl of 0.005 N HCl. The bound cAMP is eluted by flushing the resin with 3 × 200 μl of 0.1 M ammonium acetate and transferred to scintillation vials. The radioactivity was counted in a Beckman liquid scintillation counter. The enzyme activity was calculated as picomoles of cAMP produced per minute per milligram membrane protein. A fold-stimulation over the basal activity (activity in the absence of any stimulants) was determined and calcitonin-induced stimulation was compared with that produced by forskolin.

Protein kinase C (PKC) assay

Ca^{2+}-dependent protein kinase activity of PC-3M cell membranes was determined using the PKC assay system from Life Technologies (Gaithersburg, MD). The procedures provided by the manufacturer were followed. In brief, PC-3M cells, grown to 50% to 70% confluency, were treated with varying concentrations of calcitonin for 10 min. After the incubation, the cells were rinsed with ice-cold PBS and scraped into buffer A (20 mM TRIS, pH 7.5, 10 mM β-mercaptoethanol, 0.5 mM EDTA, 0.5 mM EGTA, 0.5% Triton X-100, 25 μg/ml each of aprotinin and leupeptin) and homogenized in a pre-cooled dounce homogenizer. Membrane fractions were collected after centrifugation at 60,000 g and resuspended in buffer A. The resuspended membranes were added to the assay buffer (20 mM TRIS, pH 7.5, 20 mM $MgCl_2$, 1 mM $CaCl_2$, 20 μM [γ-^{32}P]-ATP-1 nmol per assay tube) and 50 μM acetylated myelin basic protein (substrate). Reaction samples were incubated at 30°C for 5 min and transferred individually to a phosphocellulose membrane. The membranes were then washed twice with 1% phosphoric acid and twice with water. The membranes were then added to the scintillation fluid and radioactivity retained by the membranes was counted. Specific PKC activity was determined as picomoles phosphate incorporated per minute after subtracting non-specific radioactivity incorporated (radioactivity incorporated in the presence of excess synthetic PKC inhibitor). PMA (200 nM) served as a positive stimulator. The results were expressed as picomoles per minute per 20 μg membranes.

DNA constructs

cDNAs for wild-type and constitutively active Gα subunits were kindly provided by Drs. R. Iyengar (Department of Pharmacology, Mount Sinai Medical Center, NY) and J. Silvio Gutkind (National Institute of Dental Research, NIH, Bethesda, MD). cDNA constructs for constitutively active forms of Gα subunits were developed by PCR-directed mutagenesis (GTPase-inhibiting mutations Q227L for $G_s\alpha$ and Q209L for $G_q\alpha$) and subcloned in expression plasmids carrying cytomegalovirus (CMV) promoter and a dominant selectable marker, neo.[18,19] Stable transfectants were selected using geneticin (G418, GIBCO-BRL, Gaithersburg, MD).

Transfection

Plasmid DNA transfection of PC-3M prostate cancer cells was performed using Lipofectamine (Life Technologies). In brief, PC-3M cells were plated at a density of 150,000 cells per well in a 6-well culture plate and transfected 24 hr later with either plasmids carrying cDNAs for either wild-type (WT) or constitutively active (QL) Gα subunits. Aliquots containing 2 μg plasmid and 4 mg Lipofectamine (Life Technologies) in 1 ml serum-free, protein-free DMEM were incubated for 45 min and added to culture wells. The transfection media were replaced with the complete medium 16 hr later. Two days later, the cells were cultured in the selection medium (complete medium containing 400 μg/ml of G418). Individual colonies of the transfectants were selected after 4 weeks of culture, dispersed with trypsin/EDTA and propagated further into fresh flasks. The cell colonies displaying the highest expression of Gα subunits were chosen for further investigation.

Immunodetection of gα subunits

Crude membranes from the transfectants were prepared as previously described.[20] In brief, 10 million cells were homogenized in buffer A (10 mM TRIS-HCl, pH-7.4, 1 mM $MgCl_2$, 50 μg/ml aprotinin and 50 μg/ml leupeptin). Nuclear fraction and debris were separated by centrifugation at 2,000 g for 10 min at 4°C and the supernatant was centrifuged at 80,000 g for 60 min to obtain a membrane pellet. Protein concentrations of membrane suspension were determined by the method of Bradford (Bio-Rad, Hercules, CA). The membrane pellets were then solubilized by boiling in 2× Laemmli solution containing 20 mM DTT and alkylated by incubation with iodoacetamide (40 mM). Approximately 50 μg protein was loaded on a 12.5% SDS-polyacrylamide gel. The separated proteins were transferred to nitrocellulose and the blots were stained with specific antiserum against the appropriate Gα subunit.[21] The immune complexes were visualized using alkaline phosphatase-conjugated goat anti-rabbit IgG (Promega, Milwaukee, WI).

Growth curves of PC-3M transfectants

To determine the rate of cell growth, the cells (either parental, WT or QL transfected) were seeded in 24-well plates at 2 × 10^5 cells per well in 1 ml complete medium. The cells from triplicate wells were removed every day and counted in a hemocytometer. Remaining wells had their medium change d on alternate days during the course of the experiment.

cAMP accumulation

Approximately 100,000 cells (either PC-3M cells or those expressing either $G_s\alpha$-WT or $G_s\alpha$-QL) per well were seeded in 24-well plates and cultured overnight. The cells were then washed in serum-free incubation medium (RPMI 1640 supplemented with 0.3% BSA, 10 mM HEPES, 50 U/ml penicillin, 50 μg/ml streptomycin and 10 μM 3-isobutyl-1-methyxanthine or IBMX) and incubated further for 15 min in the presence/absence of 50 μM forskolin. Each treatment had at least 4 replicates. The cells were then washed with 1 ml sodium acetate buffer (pH 4.8) and lysed by repeated freeze-thawing. The lysates were centrifuged at 4°C to remove insoluble materials, and the supernatants were stored frozen at −70°C until analyzed for cAMP by RIA as previously described (Shah *et al.*, 1994). The assay used reagents ([^{125}I]2′-0-monosuccinyl-cAMP and anti-cAMP rabbit serum) from Biomedical Technologies (Boston, MA). The results are expressed as femtomolar cAMP content per 100,000 cells.

RESULTS

Calcitonin increases ^{3}H-thymidine incorporation in PC-3M cells

These experiments were conducted under 2 conditions. In the first experiment, PC-3M cells were cultured in the complete medium, washed and incubated with various concentrations of CT for 24 hr. The results in Figure 1*A* reveal that CT induced a dose-dependent increase in ^{3}H-thymidine incorporation. An increase of 53% was observed when treated with 100 nM CT, the highest dose in these experiments.

In these experiments, PC-3M cells were serum starved for 24 hr prior to the exposure to calcitonin in order to reduce their proliferative activity and synchronize their cell cycles. The results presented in Figure 1*B* reveal that calcitonin induced a dose-dependent increase in ^{3}H-thymidine incorporation of PC-3M cells. An approximately 3-fold increase in DNA synthesis was observed in response to 1 μM calcitonin, the highest tested concentration in these experiments. The experiment also tested the effects of Rp-.cAMP, a competitive inhibitor of cAMP-dependent protein kinase A, and nifedipine, a calcium channel blocker. Rp.cAMP (100 μM) shifted the calcitonin dose-response curve to the right, but calcitonin could still induce a significant increase in DNA synthesis when its concentration was 10 nM or greater. In contrast, 6 nM

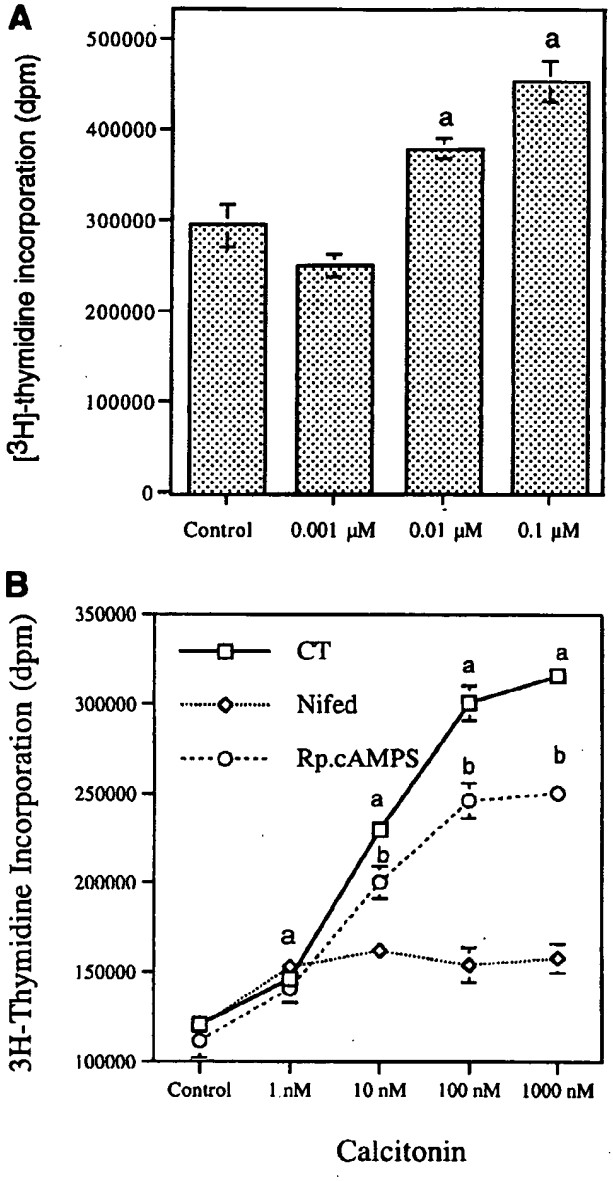

FIGURE 1 – Effect of calcitonin on ^{3}H-thymidine incorporation of PC-3M cells. (*a*) Rate of thymidine incorporation in PC-3M cells is expressed as ^{3}H-thymidine incorporated (dpm × 1,000; mean ± SEM) during 24 hr incubation with various concentrations of calcitonin and 0.1 μCi ^{3}H-thymidine in serum-free basal medium. (*b*) Rate of thymidine incorporation in PC-3M cells is expressed as ^{3}H-thymidine incorporated (dpm × 1,000; mean ± SEM) during 24 hr incubation with various concentrations of calcitonin and 0.1 μCi ^{3}H-thymidine in serum-free basal medium. The PC-3M cells were incubated for 24 hr in a serum-free basal medium for 24 hr prior to the addition of calcitonin and forskolin or nifedipine. [a,b]Significantly different from control, $p < 0.05$.

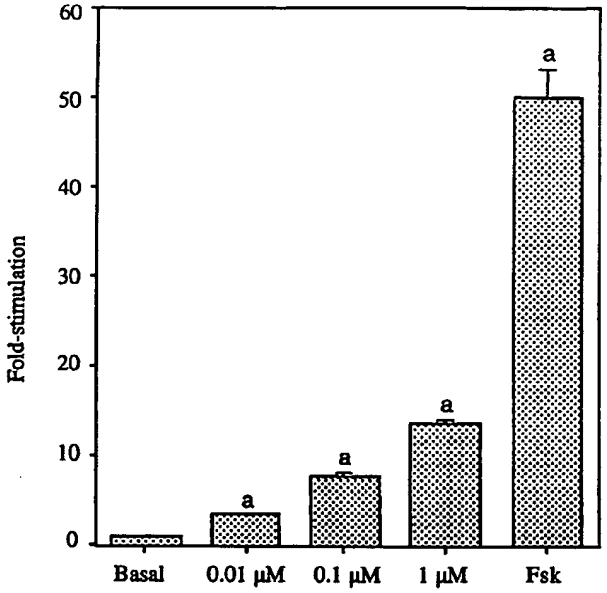

FIGURE 2 – Effect of calcitonin on adenylyl cyclase activity of PC-3M cells. Effect of calcitonin on adenylyl cyclase activity of PC-3M lysate as described in Material and Methods. The results are expressed as fold-stimulation over basal adenylyl cyclase activity of PC-3M plasma membranes. Basal adenylyl cyclase activity (in the absence of stimulants) was considered as 1. [a]Significantly different from control, $p < 0.05$.

nifedipine completely abolished calcitonin-induced DNA synthesis without affecting basal proliferative rate or viability of PC-3M cells (Fig. 1*B*).

Calcitonin stimulates adenynyl cyclase and PKC activities in PC-3M cells

Previous studies have shown that calcitonin activates Gs and Gq signaling cascades in LnCaP cells. We tested whether the activation of calcitonin receptor in PC-3M cells also leads to similar activation of these 2 mechanisms. We examined the effect of calcitonin on adenylyl cyclase and PKC activities of PC-3M cells.

The results presented in Figure 2 demonstrate that calcitonin stimulated adenylyl cyclase activity in a dose-dependent manner. An approximately 12-fold increase in the activity was observed when the cells were treated with 1 μM calcitonin. Forskolin (50 μM), a positive control, caused a much larger, over 50-fold, increase in adenylyl cyclase activity.

The experiments also examined the effect of various concentrations of calcitonin (10 nM–10 μM) on PKC activity. The preliminary experiments revealed that 0.1 μM calcitonin provided the maximal response (data not shown). Subsequent experiments used 0.1 μM calcitonin to determine the time course of calcitonin-induced PKC activation. The results presented in Figure 3 demonstrate that a 3-fold increase in the PKC activity occurred after 1 min of incubation, and this increase was sustained for up to 10 min. Following this, the activity declined and returned closer to the baseline levels in 60 min.

Transfection of Gα subunits in PC-3M cells

To test the role of $G_s\alpha$- and $G_q\alpha$-mediated signaling pathways in proliferation of PC-3M cells, stable PC-3M transfectants expressing either $G_s\alpha$-QL or $G_q\alpha$-QL were created. Parental PC-3M cells and transfectants expressing $G_s\alpha$-WT and $G_q\alpha$-WT served as controls. Membrane lysates of the transfectants were tested to confirm the increase in expression of Gα subunit proteins by Western blot analysis. As expected, PC-3M cells and $G_s\alpha$ transfectants displayed 2 major $G_s\alpha$-immunoreactive species of 50–52 kDa and 43–45 kDa sizes (Fig. 4*A*). Molecular weights of these species were consistent with those of long and short forms of $G_s\alpha$ proteins.[22] Moreover, $G_s\alpha$-immunoreactive bands in lysates prepared from transfectants (mutant as well as wild type) were more intense than those from untransfected PC-3M cells. Densitometric analyses of these immunoblots revealed that $G_s\alpha$ transfectants

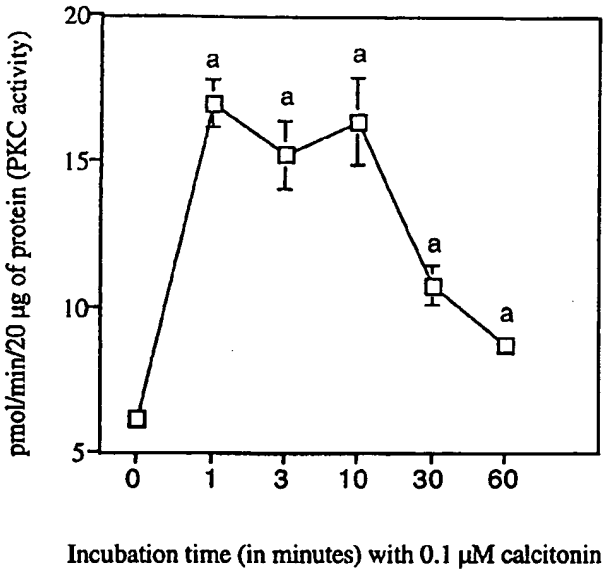

FIGURE 3 – Effect of calcitonin on PKC activity of PC-3M cells. Time course of calcitonin-induced activation of PKC. PC-3M cells were incubated with 0.1 µM calcitonin for various time points, homogenized and membranes were incubated with [γ-32P]-ATP and PKC substrate for 5 min at 30°C using a PKC assay kit (GIBCO-BRL). The results are expressed as phosphorylation pmol/minute/20 µg membrane protein. Background counts (in the presence of 10 µM synthetic PKC substrate inhibitor): 3,090 ± 469. Maximum counts (in the presence of 100 nM PMA): 55,983 ± 7076. [a]Significantly different from control; $p < 0.05$.

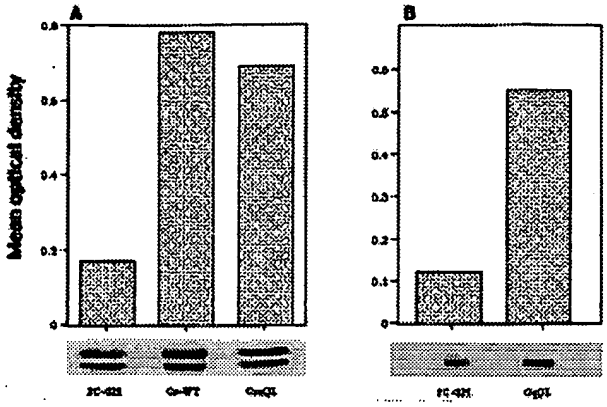

FIGURE 4 – Western blot analysis for Gα subunits in PC-3M transfectants. (*a*) Western blot analysis for $G_s\alpha$ protein in membrane lysates of PC-3M cells (untransfected): $G_s\alpha$-WT (PC-3M cells transfected with $G_s\alpha$-WT) and $G_s\alpha$-QL (PC-3M cells transfected with GTPase-deficient $G_s\alpha$). Fifty micrograms of membrane protein was loaded after denaturation and alkylation as described in Material and Methods. Two immunoreactive bands of 50–52 and 43–45 kDa are consistent with long and short forms of the $G_s\alpha$ subunit.[22] The cells transfected with either $G_s\alpha$ subunit (wild type or mutant) exhibited at least a 4-fold increase in $G_s\alpha$ immunoreactive bands (as determined by densitometry). (*b*) Western blot analysis for $G_q\alpha$ protein in membrane lysates of PC-3M (untransfected and stable $G_q\alpha$-QL transfectants). A major immunoreactive band of approximately 42 kDa was observed. The size of this immunoreactive species is consistent with the published size of the $G_q\alpha$ protein.[22] As expected, membrane lysates from $G_q\alpha$-QL transfectants contained markedly higher concentrations of $G_q\alpha$ protein.

contained 4-fold greater concentrations of $G_s\alpha$ proteins compared with untransfected PC-3M cells. Specifically, the increase in a heavy band (50–52 kDa, the protein product of the cDNA construct) was clearly observable.

As expected, $G_q\alpha$ transfectants displayed a single $G_q\alpha$-immunoreactive band of the anticipated size of ~42 kDa (Fig. 4*B*).[18] Moreover, the $G_q\alpha$ immunoreactivity in transfectant cells was at least 4-fold higher than untransfected PC-3M cells.

Expressed $G_s\alpha$ proteins in the transfectants are functional: increased cAMP accumulation

If the expressed $G_s\alpha$ proteins are functional, they should stimulate cAMP accumulation in these cells. We examined the basal (unstimulated) and forskolin-induced cAMP accumulation in PC-3M cells and $G_s\alpha$ transfectants. The results presented in Figure 5 demonstrate that the cells expressing $G_s\alpha$-WT and $G_s\alpha$-QL proteins caused an increase in basal cAMP accumulation by 11- and 85-fold, respectively. All cell lines responded to forskolin (50 µM). These results suggest that the expressed $G_s\alpha$ proteins are functional, and cause an increase in cAMP accumulation that is similar to those published in other cell culture systems.[23,24]

Expression of $G_s\alpha$-QL protein induces a dramatic increase in the growth rate of PC-3M cells

In the next experiment, we examined the growth patterns of these transfectants. 500,000 cells from each of the cell lines were seeded in a 35 mm dish and cultured for 3 days. After this period, the dishes were photographed (Figs. 6*A*–6*C*). PC-3M cells grew in monolayers (Fig. 6*A*) and $G_s\alpha$-QL transfectants displayed much faster growth. Growth in multiple layers suggested the loss of contact inhibition (Fig. 6*B*). In contrast to the accelerated proliferation of $G_s\alpha$-QL transfectants, $G_q\alpha$-QL transfectants displayed neurite outgrowth and progressive decline in cell density (Fig. 6*C*). These changes are similar to those described for PC-3M cells undergoing terminal neuroendocrine differentiation.[25] This further suggests that differential changes in PC-3M cell phenotypes introduced by the respective Gα-QL, and not WT, subunit proteins may have been predominantly due to selective activation of respective signaling pathway(s).

Because $G_s\alpha$-QL cells displayed significantly higher growth, we subsequently determined the rate of growth of these cell lines. Two thousand cells per well were seeded in multi-well plates and cultured over a period of 12 days. We harvested the cells from a number of wells every alternate day, and the cell density per well was determined. Growth curves of parental PC-3M cells, $G_s\alpha$-WT and $G_s\alpha$-QL transfectants suggest that $G_s\alpha$-QL transfectants displayed a 4- to 5-fold higher growth rate compared with either PC-3M cells or $G_s\alpha$-WT transfectants (Fig. 6*D*). Although $G_s\alpha$-QL cells displayed a faster growth rate than the other 2 cell lines as early as day 2 of the culture (earliest time point examined), the difference between them was not as dramatic as seen in the earlier experiment (Figs. 6*A*–6*C*). This discrepancy could be explained by the fact that the plating density of cells in this experiment was significantly less (2,000 *vs.* 500,000). Thus, the cells needed to reach a critical density before their growth was accelerated at a faster rate (Fig. 6*D*).

In consistence with their accelerated growth, $G_s\alpha$-QL transfectants also displayed a 3-fold increase in the rate of ^{3}H-thymidine incorporation when compared with parental PC-3M cells or those expressing $G_s\alpha$-WT (Fig. 6*E*).

Effect of 8-Br-cAMP and forskolin on ^{3}H-thymidine incorporation

If intracellular cAMP is a predominant stimulus for proliferation of $G_s\alpha$-QL cells, then membrane-permeable 8-bromo cAMP should also stimulate ^{3}H-thymidine incorporation in untransfected PC-3M cells. We tested the effects of increasing concentrations of 8-bromo cAMP (1 µM–1 mM) in the presence/absence of 10 µM 3-isobutyl methylxanthine (MIX) on DNA synthesis of PC-3M

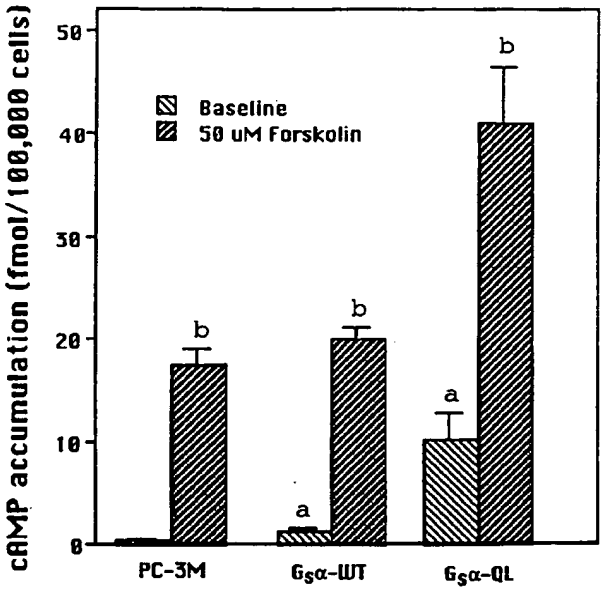

FIGURE 5 – The expressed $G_s\alpha$ proteins are functional. Basal (unstimulated) and forskolin-induced cAMP accumulation. Untransfected PC-3M cells as well those expressing either $G_s\alpha$-WT or $G_s\alpha$-QL ± 50 μM forskolin were incubated in serum-free basal medium containing 10 μM IBMX for 15 min at 37°C. cAMP concentrations in supernatants of the cell lysates were determined by RIA. Expression of either $G_s\alpha$-WT or $G_s\alpha$-QL proteins induced an 11-fold and 85-fold increase in baseline cAMP accumulation compared with parental PC-3M cells. A similar increase was also observed in forskolin response. However, forskolin-induced cAMP accumulation in PC-3M cells and those expressing $G_s\alpha$-WT were not different. $^a p < 0.05$ between PC-3M or C and $G_s\alpha$-WT or $G_s\alpha$-QL. $^b p < 0.05$ between the baseline and forskolin-stimulated groups (one-way ANOVA and Newman-Keuls test).

cells. The results presented in Figure 7*A* show that the presence of MIX caused a small, statistically insignificant increase in ^{3}H-thymidine incorporation. The addition of 8-bromo-cAMP caused a dose-dependent but much smaller increase in ^{3}H-thymidine incorporation of PC-3M cells. Although the increase produced by 100 μM or greater concentrations of 8-bromo cAMP was significantly different from the controls, it was not as dramatic as seen in $G_s\alpha$-QL transfectants.

We tested the effect of forskolin (50 μM) on ^{3}H-thymidine incorporation of PC-3M cells because forskolin directly activates adenylyl cyclase and increases intracellular cAMP levels of PC-3M cells to the levels comparable to $G_s\alpha$-QL cells (see Fig. 5). Similar to 8-Br-cAMP, forskolin also caused a significant but smaller increase in ^{3}H-thymidine incorporation (Fig. 7*B*).

To further examine the role of cAMP signaling pathway in proliferation of $G_s\alpha$-QL cells, we tested the effect of 100 μM Rp.cAMP on the rate of ^{3}H-thymidine incorporation. The data presented in Figure 7*C* suggest that Rp.cAMP, a competitive inhibitor of cAMP-dependent protein kinase A, caused a small but significant decline in the rate of DNA synthesis of $G_s\alpha$-QL cells. These results are consistent with the findings presented in Figures 7*A,B* and suggest that $G_s\alpha$-QL–induced cell growth may be predominantly mediated by cAMP-independent mechanisms.

Effect of Ca^{2+} channel antagonists on ^{3}H-thymidine incorporation

Because $G_s\alpha$ has been implicated in the activation of voltage-gated Ca^{2+} channels, we tested whether the blockade of these channels will attenuate $G_s\alpha$-QL–mediated growth of PC-3M cells. Effects of nifedipine and verapamil were tested on DNA synthesis of parental PC-3M, $G_s\alpha$-WT and $G_s\alpha$-QL cells. Only $G_s\alpha$-QL, and not PC-3M or $G_s\alpha$-WT, cells displayed high sensitivity to nifedipine. At nanomolar concentrations, nifedipine caused a dramatic and dose-dependent decline in the rate of ^{3}H-thymidine incorporation of $G_s\alpha$-QL cells (Fig. 8). Nifedipine 6 nM was sufficient to almost abolish DNA synthesis in $G_s\alpha$-QL cells. Nifedipine-mediated decline in DNA synthesis was not due to apoptotic events because nifedipine (6 nM)-treated cells displayed 93% viability as assessed by the trypan blue exclusion test. These experiments were repeated with 3 separate batches of $G_s\alpha$-QL cells, and similar results were obtained. The results also revealed that nanomolar concentrations of nifedipine did not affect the rate of DNA synthesis of parental PC-3M and $G_s\alpha$-WT cells (Fig. 8). Verapamil was much less effective and required micromolar concentrations to inhibit DNA synthesis of $G_s\alpha$-QL cells. However, this effect was not $G_s\alpha$-QL cell specific, but inhibited DNA synthesis of all cell lines at these concentrations (Fig. 9).

DISCUSSION

Similar to the previously reported actions on indolent LnCaP prostate cancer cells,[9] calcitonin also induced mitogenic responses in highly invasive PC-3M cells. These results, when combined with the reports of increased expression of calcitonin in PC,[8] support the hypothesis that calcitonin is an important paracrine/autocrine growth factor that plays an important role in androgen-independent tumor growth. PC-3M cells have been derived from liver metastasis of PC-3 transplanted prostate tumor in nude mice.[14] However, they differ in several major characteristics from parental PC-3 cells. For example, PC-3 cells are androgen refractory but not very aggressive. In contrast, PC-3M cells are androgen refractory and highly invasive.[14] Unlike PC-3 cells, PC-3M cells express neuroendocrine factors and undergo neuroendocrine differentiation under experimental conditions.[25] Our results reveal that PC-3M cells respond to calcitonin with mitogenic activity. In contrast, PC-3 cells have been reported to be unresponsive to calcitonin.[10] The calcitonin-induced dual activation of adenylyl cyclase- and PKC-mediated signaling in PC-3M cells is similar to that observed in LnCaP cells.[9]

Because calcitonin activated $G_s\alpha$- and $G_q\alpha$-mediated signaling, the second objective of our study was to test the role of these G-proteins in proliferation of prostate cancer cells. The results have shown that constitutive expression of $G_s\alpha$ subunit in PC-3M cells caused a dramatic change in their growth characteristics as indicated by a large increase in proliferation and saturation density, and a loss of contact inhibition. These changes may have been specifically caused by activated $G_s\alpha$ subunits because the transfectants expressing either wild-type $G_s\alpha$ or constitutively active $G_q\alpha$ did not display similar changes. In contrast, $G_q\alpha$-QL cells exhibited morphological characteristics of terminally differentiated cells. These results suggest that the $G_s\alpha$-mediated signaling cascade plays a prominent role in proliferation of PC-3M prostate cancer cells. They are consistent with previous reports that inclusion of cholera toxin, an activator of $G_s\alpha$, in the culture medium is critical for the propagation of primary prostate epithelial cells in culture.[26] There is other evidence to support the role of $G_s\alpha$ in tumorigenesis or progression of various human carcinomas.[23,27–29] For example, agents or genomic mutations activating intracellular cAMP accumulation stimulate proliferation of thyrocytes, pituitary somatotropes, lung cancer cells, primordial germ cells and prostate cancer LnCaP cells.[12,30–32] Dibutyryl cAMP increases *in vitro* migration rate and invasiveness of LnCaP cells.[28] cAMP-dependent protein kinase A, a down-stream effector of cAMP cascade, increases the invasiveness and metastasizing ability of lung cancer cell lines.[33] Somatic mutations in the $G_s\alpha$ gene that cause the formation of constitutively active $G_s\alpha$ (referred as *gsp* oncogene) have been detected in a variety of human diseases including pituitary and thyroid tumors.[24,27,30] However, the occurrence of these mutations in prostate adenocarcinoma has not been extensively investigated.

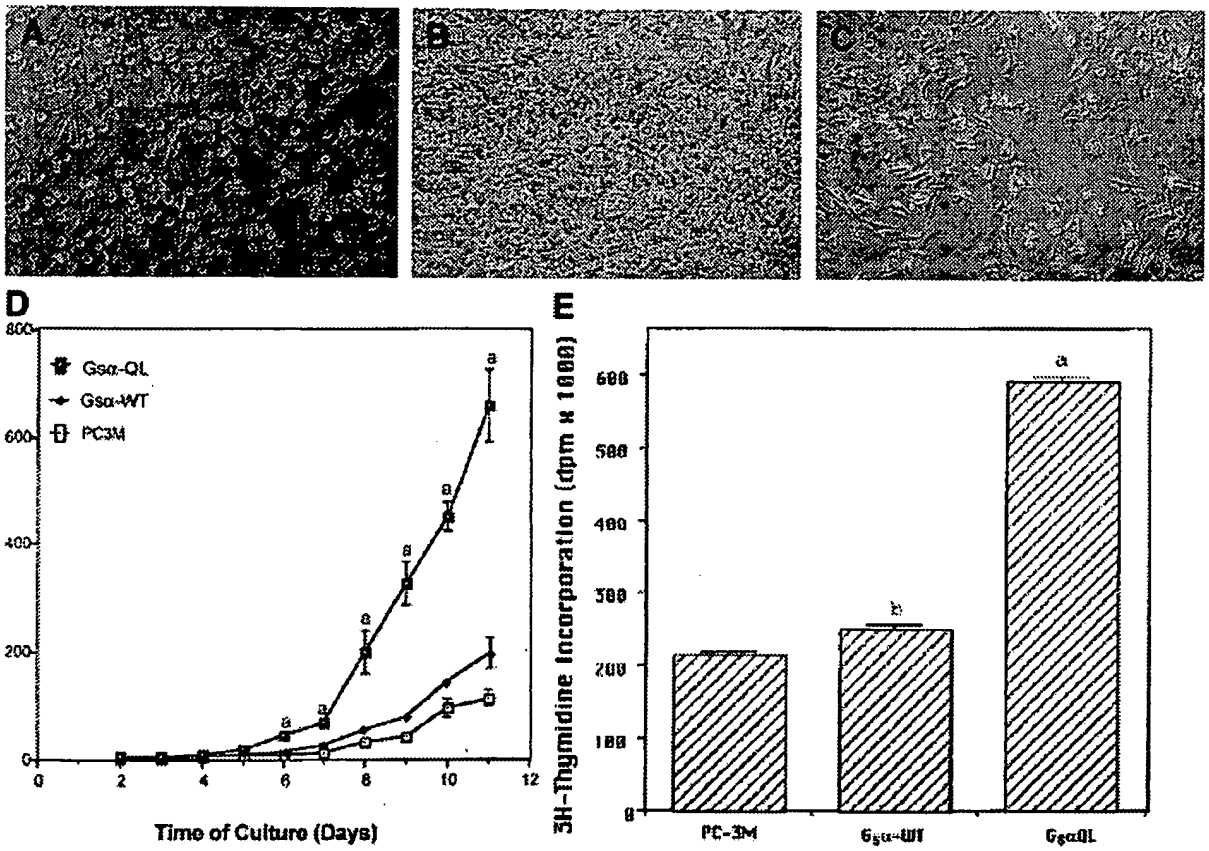

FIGURE 6 – Expression of $G_s\alpha$-QL increases proliferation of PC-3M cells. (*a*) A photomicrograph showing cultures of PC-3M cells 3 days after seeding. Approximately 500,000 cells in a 35 mm dish were plated. (*b*) A photomicrograph showing cultures of $G_s\alpha$-QL transfectants after 3 days of culture. Approximately 500,000 cells in a 35 mm dish were plated. Markedly denser, multilayered growth (compared with Fig. 1*b*) is evident. (*c*) A photomicrograph showing cultures of $G_s\alpha$-QL transfectants after 3 days of culture. Approximately 500,000 cells in a 35 mm dish were plated. Decreased cell density (compared with Fig. 1*b*) and gross changes in morphology (neurite outgrowth) are evident. (*d*) Expression of $G_s\alpha$-QL increases cell proliferation. A comparison of the rate of cell proliferation of parental PC-3M cells, $G_s\alpha$-WT and $G_s\alpha$-QL transfectants. Two thousand PC-3M cells and $G_s\alpha$-QL transfectants were seeded per well and cultured over a period of 12 days as described in Material and Methods. The results are expressed as number of cells per well after days of initial plating. $^a p < 0.05$ (one-way ANOVA and Newman-Keuls test). (*e*) Expression of $G_s\alpha$-QL increases DNA synthesis. Rate of thymidine incorporation in untransfected PC-3M cells, stable transfectants expressing $G_s\alpha$-WT and $G_s\alpha$-QL is expressed as ^{3}H-thymidine incorporated (dpm × 1,000) during 24 hr incubation with 0.1 μCi ^{3}H-thymidine in serum-free basal medium. Significantly different from PC-3M cells. $^a p < 0.05$ between PC-3M and $G_s\alpha$-QL or $G_s\alpha$-WT cells. $^b p < 0.05$ between $G_s\alpha$-QL and $G_s\alpha$-WT (one-way ANOVA and Newman-Keuls test).

The mechanism by which calcitonin or $G_s\alpha$-QL stimulates proliferation and DNA synthesis of PC-3M cells is not clear. To test whether these actions are mediated through the cAMP signaling cascade, we examined the effects of 8-Br-cAMP and forskolin on DNA synthesis of PC-3M cells. Both these agents caused a dose-dependent, but much smaller, increase in ^{3}H-thymidine incorporation. Conversely, we tested whether blockade of the cAMP signaling cascade would attenuate the proliferative activity of $G_s\alpha$-QL transfectants or calcitonin-treated PC-3M cells. We examined the effect of Rp.cAMP on DNA synthesis of these cells. Although high concentrations of Rp.cAMP (100 μM) were able to shift the calcitonin response curve to the right, calcitonin could still significantly stimulate DNA synthesis of PC-3M cells. Similarly, Rp.cAMP caused only a small decrease in the rate of ^{3}H-thymidine incorporation in $G_s\alpha$-QL cells. Therefore, it is highly likely that mitogenic actions of $G_s\alpha$-QL in PC-3M cells are predominantly mediated by adenylyl cyclase-independent mechanisms.

$G_s\alpha$ has also been shown to directly activate voltage-gated Ca^{2+} channels in a variety of cell types.[34,35] The role of cytoplasmic Ca^{2+} in cell proliferation has been extensively documented.[36,37] Verapamil and nifedipine belong to the aphenylalkyamine and dihydropyridine class and are well-characterized antagonists of voltage-gated Ca^{2+} channels. They are generally believed to inhibit plasma membrane-bound calcium channels. In addition, these drugs are suggested to affect several intracellular processes such as modulation of P-glycoprotein–induced drug transport, inhibition of PKC, calmodulin or phosphodiesterases. To test the role of voltage-gated calcium channels in calcitonin or $G_s\alpha$-QL–induced cell proliferation, we investigated whether nifedipine (or verapamil) attenuate the $G_s\alpha$-QL–induced increase in DNA synthesis. The results have shown that nifedipine did not affect DNA synthesis of either untreated PC-3M cells or $G_s\alpha$-WT transfectants, but selectively blocked the DNA synthesis of calcitonin-treated PC-3M cells as well as $G_s\alpha$-QL transfectants. On the other hand, verapamil attenuated DNA synthesis in all cell lines, but only when used at a thousand-fold greater concentration (compared with nifedipine). Because nifedipine and verapamil are both effective in blocking voltage-gated Ca^{2+} channels, it is likely that the influx of extracellular Ca^{2+} plays an important role in proliferation of all cell lines. However, nifedipine, and not verapamil, was effective in selective blocking of $G_s\alpha$-QL–induced mitogenesis.

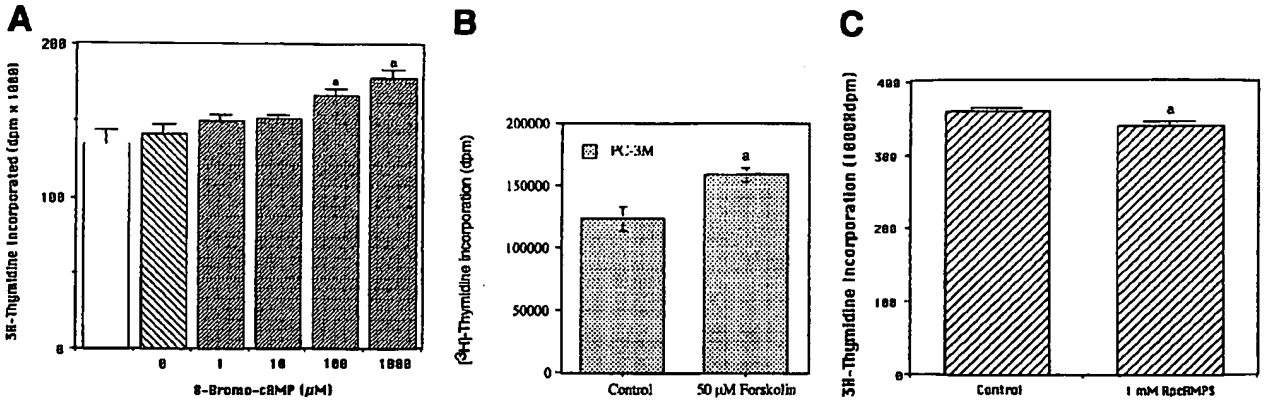

FIGURE 7 – Effect of cAMP in PC-3M DNA synthesis. (*a*) Effect of 8-Br-cAMP on ^{3}H-thymidine incorporation in PC-3M cells. 30,000 PC-3M cells were plated in multi-well culture plates and incubated with 0.1 μCi ^{3}H-thymidine in the presence or absence of 10 μM IBMX and/or various concentrations of 8-Br-cAMP. IBMX caused a slight increase in ^{3}H-thymidine incorporation (hatched bar with 0 8-Br-cAMP). 8-Br-cAMP caused a smaller but dose-dependent increase in ^{3}H-thymidine incorporation. The increase produced by 100 μM produced greater than 50% increase compared with the vehicle control. [a]Significantly different from control ($p < 0.05$; one-way ANOVA and Newman-Keuls test). (*b*) Effect of forskolin on ^{3}H-thymidine incorporation of PC-3M cells. 30,000 PC-3M cells were plated in multi-well culture plates and incubated with 0.1 μCi ^{3}H-thymidine in the presence or absence of 50 μM forskolin. Forskolin caused approximately 20% increase in the rate of DNA synthesis. [a]Significantly different from control ($p < 0.05$, one-way ANOVA and Newman-Keuls test). (*c*) Effect of Rp.cAMP on DNA synthesis of $G_s\alpha$-QL transfectants. Effect of 1 mM Rp.cAMP on ^{3}H-thymidine incorporation of $G_s\alpha$-QL transfectants was examined as described in Material and Methods. The results are expressed as ^{3}H-thymidine incorporated (dpm × 1,000) per 300,000 cells per 24 hr. Rp.cAMP caused a small but significant decline in ^{3}H-thymidine incorporation of $G_s\alpha$-QL transfectants. [a]Significantly different from control, $p < 0.05$ (one-way ANOVA and Newman-Keuls test).

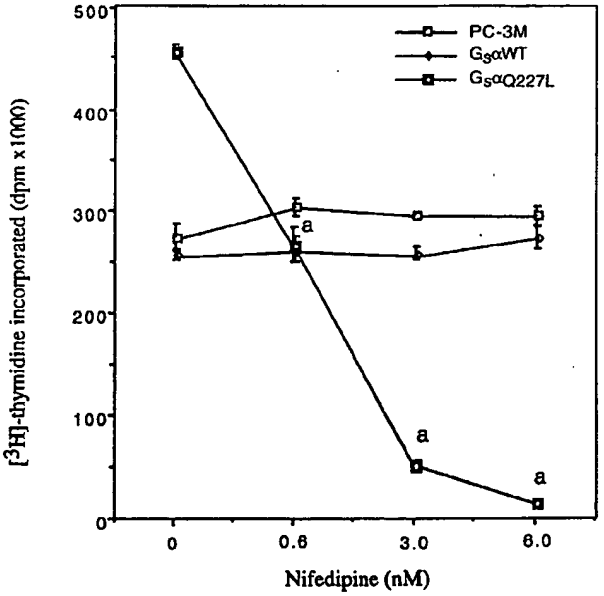

FIGURE 8 – Effect of nifedipine on ^{3}H-thymidine incorporation. PC-3M cells as well as $G_s\alpha$-WT and $G_s\alpha$-QL transfectants were treated with various concentrations of nifedipine. Its effects on ^{3}H-thymidine incorporation were examined. Nifedipine caused a dose-dependent attenuation of ^{3}H-thymidine incorporation of $G_s\alpha$-QL transfectants. However, it did not affect DNA synthesis of PC-3M cells and $G_s\alpha$-WT transfectants. [a]Significantly different from control ($p < 0.05$; one-way ANOVA and Newman-Keuls test).

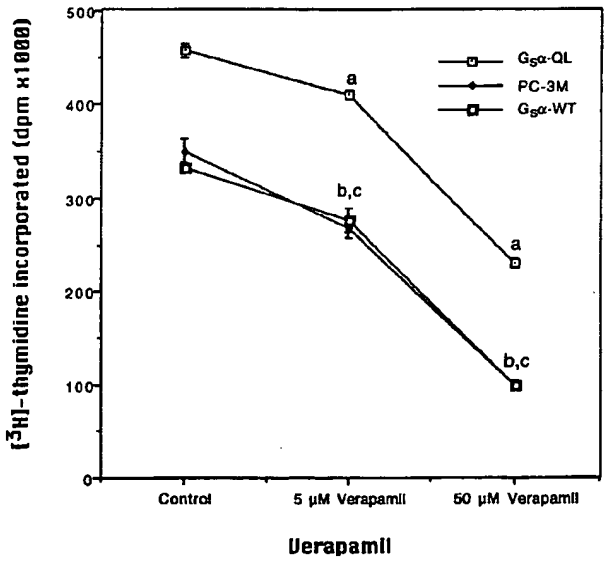

FIGURE 9 – Effect of verapamil on ^{3}H-thymidine incorporation. PC-3M cells as well as $G_s\alpha$-WT and $G_s\alpha$-QL transfectants were treated with 5 and 50 μM of verapamil as described in Material and Methods. Its effect on ^{3}H-thymidine incorporation was evaluated. Verapamil caused a significant decrease in ^{3}H-thymidine incorporation of all cell lines. [a]Significantly different from control ($p < 0.05$, one-way ANOVA and Newman-Keuls test). [b,c]Significantly different from control ($p < 0.05$, one-way ANOVA and Newman-Keuls test).

Therefore, it is highly unlikely that this process is mediated by voltage-gated Ca^{2+} channels alone. It is more likely that $G_s\alpha$-QL selectively activates other events associated with PC-3M cell proliferation, and nifedipine selectively blocks these processes.

In addition to antagonization of voltage-gated Ca^{2+} channels, nifedipine selectively affects several other processes associated with cell proliferation.[38–42] Other events associated with G-protein–induced cell proliferation include an increase in oscillations of intracellular Ca^{2+}, leading to the activation of Ca^{2+}-sensitive K^+ channels, an increase in cell size and marked reorganization of

the cytoskeleton.[42,43] The activation of these pathways leads to activation of Rho, rearrangement of the actin cytoskeleton and depolymerization of actin containing stress fibers.[42,43] It has been shown that only nifedipine, but not verapamil, selectively blocks some of these processes such as an increase in oscillations of intracellular Ca^{2+}, an increase in cell volume and cytoskeletal rearrangement.[42] Considering the present findings that nifedipine, and not verapamil, was selective in blocking $G_s\alpha$-QL–induced cell proliferation, it is very likely that this involves nifedipine-sensitive events such as an increase in oscillations of intracellular Ca^{2+} and rearrangement of the cytoskeleton. Additional studies will be necessary to delineate these events.

The present findings that $G_s\alpha$-mediated intracellular mechanisms cause a dramatic change in growth characteristics of prostate cancer cells could be of significance in the pathophysiology of PC. There is evidence to suggest that several neuropeptides are synthesized and released by prostate cancer cells, and may function as principal mitogens in these tumors.[9,12,44,45] Among these, calcitonin-like, VIP-like, TSH-like peptides as well as their $G_s\alpha$-coupled receptors have been localized in the prostate epithelium.[12,46] This evidence, when coupled with the present results that constitutively active $G_s\alpha$ causes a dramatic increase in proliferative activity of PC-3M cells, suggests that either the presence of the *gsp* oncogene or persistent stimulation of $G_s\alpha$-coupled receptors through autocrine/paracrine actions of their ligands could significantly accelerate growth of PC tumor cells. This could contribute to the progression of PC in the androgen-independent phase of the tumor.

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